Chapter 3

Effect of plant genotype and growth stage on the ß-proteobacterial community associated with different potato cultivars in two fields

Ö. İnceoğlu, J. Falcão Salles, L. van Overbeek and J.D. van Elsas (2010). *Applied Environmental Microbiology* **76**(11):3675-3684.

Abstract

Bacterial communities in the rhizosphere are dynamic and susceptible to changes in plant conditions. Among the bacteria, the *ß-proteobacteria* play key roles in nutrient cycling and plant growth promotion, and hence the dynamics of their community structures in the rhizosphere should be investigated. Here, the effects of plant cultivar, growth stage and soil type on the communities associated with the potato cultivars Aveka, Aventra, Karnico, Modena, Premiere and Désirée were assessed in two different fields, containing either high- or low-organic sandy soils. Thus, bacterial and ß-proteobacterial PCR-DGGE analyses were performed to analyze plant cultivar and growth effects on the rhizospheric community structures. The analyses showed that all cultivars in both fields exerted a rhizosphere effect on the total bacterial as well as ß-proteobacterial communities. In addition, plant growth stage strongly affected the ß-proteobacterial communities in both fields. Moreover, the community structures were affected by cultivar type, which were different in their physiologies as reflected in their growth rates, root development and estimated tuber starch contents. Betaproteobacterial clone libraries constructed for two selected cultivars (one yielding low-starch-content, the other one high-starch-content tubers) as well as bulk soil revealed that the rhizospheres of both cultivars selected for specific bacteria, under which plant-growth-promoting bacteria such as *Variovorax* and *Achromobacter* spp. In addition, qPCR-based quantification of the *Variovorax paradoxus* specific functional gene *asfA* (involved in desulfonation) indicated clear potato rhizosphere effects on the abundance of this gene. Interestingly, both cultivar type and plant growth stage affected the community under particular circumstances.

Introduction

Soil-borne microbial communities are influenced by plant roots due to, among other factors, the excretion of organic compounds in the exudates. Plants thus selectively attract microorganisms in their rhizospheres which then consume particular excreted compounds [9, 13]. It clearly follows that microbial communities in the rhizosphere may be differentially influenced by plant genotype as well as developmental stage if these incur different patterns of root exudation [12, 14, 53]. However, there is a paucity of knowledge on the extent to which such community shifts may occur, on the dynamics of the changes and on the putative effects regarding the functioning of the system.

Currently, several initiatives have been deployed to produce novel crop plants (genetically modified or obtained via traditional breeding) that contain genetic systems which enable them to control bacterial and/or fungal pathogens [19, 27, 41]. Other crop plants have been developed for diverse industrial production purposes (e.g. potato plants yielding tubers with low amylose content for the paper industry) [21]. Given the fact that it is, in most cases, unknown whether and to which extent the novel crops affect the microbial communities in soil, it is important to assess these effects in relation to effects of currently-used cultivars. This comparative assessment would enable the description of effects of novel cultivars in the context of those of existing ones, yielding a dataset that establishes a baseline describing crop plant effects on the living soil. In particular the impact on the soil's life support functions (LSF), such as biogeochemical cycles and plant health support, would be crucial to establish.

A sensible strategy to approach the effect of plants on the soil LSF is to focus on a limited set of key organisms involved in several processes. One such key group is represented by the ß-proteobacteria, as members of this group are important mediators in the cycling of nitrogen, sulfur and carbon through the soil ecosystem [25, 46]. For instance, the *Nitrosomonadaceae* form an important cluster of ß-proteobacterial ammonia oxidizers and *Burkholderia* types play important roles in the mycorrhization of plants as well as in symbiotic nitrogen fixation [3, 42]. Other members of the *ß-proteobacteria* promote plant growth by virtue of their synthesis of phytohormones and vitamins [8]. For instance, *Burkholderia phytofirmans* typically produces aminocyclopropane-1-carboxylate (ACC) deaminase, which assists in the lowering of the level of the stress hormone ethylene at plants. Accordingly, the growth of plant roots may increase when *B. phytofirmans* is present, by virtue of the reduction of this root elongation inhibitor [47]. Furthermore, other *Burkholderia* species are important producers of antibiotics that antagonize bacterial and fungal phytopathogens [28]. Recently, the ß-proteobacterial genera *Variovorax* and *Polaromonas* were found to be capable of desulfonating aromatic sulfonates in the wheat rhizosphere [45]. Since the aforementioned *ß-proteobacteria* are key drivers in the respective LSF of soil, they can provide good indicators to assess the impacts on the respective soil processes that may be exerted by novel crops. In particular the dynamics of desulfonating bacterial communities is important, as freely available sulfur (e.g. sulfate) can be limiting in plant nutrition [23, 46]. We thus hypothesized that the ß-proteobacterial types which perform key tasks like desulfonation may be differentially selected by the rhizospheres of different potato cultivars. Therefore, in this study we assessed, next to total bacteria, the dynamics of ß-

proteobacterial communities with different potato cultivars over a growth season in two soils. We focused on the extent to which these cultivars differentially "sampled" the soil microbiota and how the resulting communities shifted over a growth season.

Material and Methods

Soils and soil sampling. Two experimental fields - Buinen ([B], 52°55'N-6°49E) and Valthermond ([V], 52°50'N-6°55E)- in Drenthe, The Netherlands were selected for the experiments. These fields contained divergent soil types, i.e. B – loamy sand, 5% OM (pH 5.0), V – sandy peat, 25% OM (pH 5.0). The fields were under agricultural rotation. In the previous season, spring barley had been grown in both fields. Six different potato cultivars (Aveka [A], Aventra [Av], Karnico [K], Modena [M; modified from Karnico for low amylose content] [7], Premiere [P] and Désirée [D]) were used. Cultivars A, Av, K and M produced tubers with high starch contents and had a low and/or medium growth rate, whereas cultivars P and D yielded tubers with relatively low starch contents and had high growth rate. The different cultivars had different parental cultivars in the first generation, so that their overall pedigree was complex. For instance, cultivar A was related to D in the fifth generation and to K in the third generation [52].

For each potato cultivar, four replicate plots randomly distributed over the fields were used. At the start of the growth season, these plots were cropped with twenty plants (tubers) each. The fields were under standard agricultural practice. Samples were taken at the young plant (EC30), flowering (EC60) and senescence stages (EC99) [18, 34]. The bulk soil sampling moments will be referred to as June, July and September in the rest of the text.

At each time, both plants and bulk soil were sampled. Per plot, four replicate plants were obtained and taken to the laboratory. The loosely adhering soil on the roots was shaken off, and the resulting roots (containing rhizosphere soil) were pooled per plot, followed by collection of the rhizosphere by brushing off the soil tightly adhering to the root surface. Besides, six composite bulk soil samples, each consisting of four cores of each cultivar area outside of the reach of plant roots, were collected. Twenty-four composite samples of each treatment were thus obtained per sampling time and per field. A total of 180 composite samples were taken over the growth season, including bulk soil samples. Besides, bulk soil samples were taken before planting and after harvesting. All samples were stored in closed plastic bags (containing 1 volume headspace) at room temperature for < 2 days prior to soil DNA extraction and analysis.

Soil DNA extraction. Pooled samples of bulk soil were used directly for DNA extraction, whereas rhizosphere soil was pooled per plot as one replicate of four. For soil DNA extraction, the Powersoil DNA extraction kit (Mo Bio Laboratories Inc., NY, USA) was then used with 0.5 g of soil according to the manufacturer's instructions, slightly modified as follows. Glass beads (0.1mm diameter; 0.25g) were added to the soil slurries, and the cells were disrupted by bead beating (mini-bead beater; BioSpec Products, USA) three times for 60 s. To assess quantity and degree of purity, the crude DNA extracts were run on 1.5% agarose gels at 90V for 1h in 0.5x Tris-acetate-EDTA (TAE) buffer (20mM Tris, 10mM acetate, 0.5 mM EDTA; pH 8.0) using fixed amounts (5 µl,) of the 1-kb DNA ladder

(Promega, Leiden, The Netherlands) as the molecular size and quantity marker. Gels were stained with ethidium bromide for 20 min (1.2 mg/l ethidium bromide in 0.5xTAE). The quantity of extracted DNA was estimated using comparison to the ladder. DNA quality (average molecular size and purity) and quantity were estimated from gel, using the degree of DNA shearing (average molecular size) as well as the amounts of co-extracted compounds (quality) and a comparison to known DNA amounts (quantity) in the marker.

Quantitative PCR. Quantification of 16S bacterial rRNA genes was performed with primers 341F and 518R according to [37], using an annealing temperature of 55°C and no added betaine. Standards (10^4-10^8 molecules per reaction) were prepared using PCR products from the *Variovorax paradoxus* type strain DSM30034. Statistical analyses (T tests) were performed to assess the significance of the differences between the target gene numbers in the different cultivars and growth stages.

Quantitative PCR (qPCR) of *Variovorax* sp. *asfA* genes was performed using primers asfA_Varx_F1 (CTGTCGGGCATGGAGTTCT) and asfA_Varx_R1 (AGCGTCACCGGAAAGTGCT) to yield 302-bp *asfA* gene products, as described by Schmalenberger et al. [45]. The reaction mixture contained 5 ml of DyNamo capillary SYBR Green qPCR master mix (Finnzymes, Helsinki, Finland), 1 M betaine, 0.3 pmol of forward and reverse primers (asfA_Varx_F1 and asfA_Varx_R1 respectively) and 5 ng of template DNA in a total volume of 10 µl. Polymerase chain reaction conditions were 95°C for 10 min, followed by 40 cycles of 15 s 95°C, 20 s at 60°C and 20 s at 72°C [45].

PCR amplifications for denaturing gradient gel electrophoresis (DGGE) community fingerprintings. On the basis of DNA extracted from the rhizosphere and bulk soils, PCR amplifications targeting the 16S ribosomal RNA (rRNA) genes of total bacteria and ß-proteobacteria were run. Total bacterial communities were assessed by PCR directly on soil-extracted DNA. Briefly, the PCR reaction mixtures for bacterial DGGE contained 5 µl PCR buffer (60 mM Tris-HCl, 15mM $(NH_4)_2SO_4$, 5.5mM $MgCl_2$, pH 9.0), 0.5 µl formamide, 0.5 µg T4 gene 32 protein (Roche, Almere, the Netherlands), 10 nmol of each deoxyribonucleoside triphosphate, 10 pmol of each primer (GC-341F and 518R) [37] and 5U AmpliTaq® DNA polymerase, Stoffel fragment (Applied Biosystems, Foster, CA) combined with pure water to 25 µl. After addition of about 5 ng of template DNA, the mixtures were placed in a GeneAmp® PCR system 9700 cycler (Applied Biosystems, Foster, CA, USA) and thermal cycling was performed as follows: initial denaturation - 5 min at 94ºC; 35 cycles consisting of 1 min at 94ºC, 1 min at 57ºC and 2 min at 72ºC; extension for 30 min at 72ºC. A nested PCR approach was used for amplification of the ß-proteobacterial communities. Initial amplification was done with primers 27F and 865R in a touchdown protocol (30 cycles) as described by Cunliffe et al.[5]. This PCR was followed by a second (fresh) PCR reaction, in which 1 µl (5 ng) of primary PCR product was used as template DNA with DGGE primers GC-341 and 518R (25 cycles).

DGGE community fingerprintings. All DGGE profiles were generated in the Ingeny Phor-U system (Ingeny International, Goes, the Netherlands). The PCR products obtained from the soil DNAs, at estimated concentrations of 200 ng, were loaded onto polyacrylamide gels [6% (wt/vol) acrylamide in 0.5x Tris-acetate-EDTA (TAE) buffer (2.42 g Tris-base, 0.82 g

sodium acetate, 0.185 g EDTA, H_2O 1L). The bacterial and ß-proteobacterial amplicons were run on 35-65% denaturant gradient gels at 100V for 16h at 60°C. All gels were silver-stained [20] and air-dried, after which they were digitized for further analyses.

Computer-assisted analysis of DGGE fingerprints. The DGGE profiles in the different gels were stored as TIFF files. Images were normalized using the markers and the patterns subsequently compared by using clustering methods. Similarity matrices consisting of defined numbers within each gel, e.g. nine different samples in triplicate were generated using Pearson's correlation coefficient (r). Subsequently, the patterns were clustered using the unweighted pair group method with arithmetic averages (UPGMA) with GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium). Besides, data derived on the basis of Jaccard correlation, a band-based analysis, were used for redundancy analysis (RDA) using CANOCO (Microcomputer Power, Ithaca, NY). Community similarities based on relative band intensities and position were analyzed by performing canonical correspondence analyses with Monte Carlo permutation tests (CANOCO 4.0, Microcomputer Power). The Monte Carlo tests were based on 199 random permutations of the data to establish statistical significance. Moving window analyses (MWA) were used to calculate the rate-of-change parameter (Δt) for bulk soil during the season. First, the similarities of the densitometric curves of DGGE patterns were calculated based on the Pearson correlation coefficient. The percentage change (%change= 100 - %similarity) was recalculated as explained by Marzorati et al. [30].

Cloning and sequencing of ß-proteobacterial 16S rRNA gene amplicons generated from selected samples. Three clone libraries of ß-proteobacterial gene fragments were generated (using primers 27F and 865R) to cross-compare the bulk soil and cultivar A and P rhizosphere ß-proteobacterial communities. The amplicons were ligated into pGEM®-T easy vectors (pGEM®242-T Vector System II, Promega, Madison, WI, USA), followed by introduction into competent *Escherichia coli* JM109 cells by transformation according to the manufacturer's instructions. White colonies were picked and replated on LB agar plates for a second check. The samples were sequenced by AGOWA (Berlin, Germany).

Analysis of ß-proteobacterial gene sequence diversity. Prior to analyses of the sequences, these were checked for chimera formation using Bellerophon v.3 (http://greengenes.lbl.gov). Totals of 84, 115 and 121 sequences were thus obtained from Av, P and bulk soil, respectively. The sequences were classified using the Ribosomal Database Project II (RDP) classifier with a confidence threshold of 80% (http://simo.marsci.uga.edu). To determine the closest phylogenetic relatives of these sequences, BLAST-N was used with the non-redundant NCBI database. Sequence alignments and tree building were carried out using the Molecular Evolutionary Genetics Analysis (MEGA) software package [51] using the Kimura two-parameter algorithm [24] with bootstrap tests of inferred phylogeny with 1000 replications. Pairwise sequence similarities were calculated with DNADIST (http://cmgm.stanford.edu/phylip/dnadist.html) using the Kimura two-parameter algorithm [24]. On the basis of the generated similarity matrix, the sequences were assigned to operational taxonomic units (OTUs) using DOTUR [44]. The frequency data

assigned to an OTU at the "species" (97% similarity criterion) and "genus" (95% similarity criterion) levels were used to yield rarefaction curves, and Chao1 richness estimates. Sequences were also subjected to library shuffling analysis using LIBSHUFF [49] to determine if the clone libraries are significantly different.

Deposition of sequences. The sequences generated in this study were deposited in Genbank under numbers GU472842-GU473161.

Results

Plant development over the growth season. No signs of disease or nutrient limitation were seen in the potato cultivars that grew over the entire growth season (2008) in all plots of the two soils. For all cultivars and in both soils, the young plant stage (EC30) occurred around 30 days post-planting (dpp), i.e. end of June. However, cultivars A, Av, M, K on the one hand, and P and D on the other hand, showed different subsequent growth rates in both soils. The flowering stages (EC60) occurred between 50 and 60 dpp for cultivars D and P and between 80 and 85 dpp for cultivars A, Av, K and M (July). Finally, the senescence stages (EC99) were between 110 and 115 dpp for P and D, between 135 and 140 dpp for A and between 145 and 150 dpp for Av, K and M. Interestingly, cultivars P and D had produced shorter roots that, at flowering stage, averaged about 15 cm in length, whereas all other cultivars had root systems with lengths around 25 cm.

Dynamics of bacterial abundance in bulk and rhizosphere soils as assessed by qPCR. The abundance of the bacterial populations in the different samples over the growth season was estimated on the basis of bacterial 16S rRNA gene abundance measurements using qPCR. In the two bulk soils over the season, these gene copy measurements yielded fairly stable (statistically similar) target numbers, i.e. in the range $3x10^7$ to $8x10^8$ gene copies / g soil in both bulk soils. However, specific trends were observable in both soils. The gene copy numbers first decreased, albeit insignificantly ($P>0.05$), from the start until June in both fields. Following this, these numbers progressively increased until the end of the growth season (Fig –S1A).

In respect of the bacterial abundances in the rhizosphere, different trends were observed per cultivar over time in the two fields. In the B soil, significant rhizosphere effects on total bacterial abundances were observed for cultivars A, M, P and D in the young plant stage and for all cultivars in the flowering stage. In addition, we also found significant effects of the rhizosphere for cultivars Av, K, M, D in the senescence stage. Considering the dynamics over time, the total bacterial abundances increased from the young plant to flowering stages and then decreased to the senescence stage for cultivars A, Av, K and M (significant, except for cultivar A). In contrast, the bacterial abundance at cultivar D showed a significant increase from the flowering to senescence stage (Fig. 1).

In the V soil the bacterial abundance analyses showed different trends. In most cases, no significant rhizosphere and/or cultivar effects were found at the different growth stages. However, the rhizosphere community abundances increased, insignificantly, over time at cultivars A, M and decreased at cultivars P and D, whereas their abundance was roughly stable for cultivars Av and K (data not shown). The range was $5x10^8$ to $4x10^9$ in

the young plant, $1.5x10^9$ to $4.2x10^9$ in the flowering and $3x10^9$ to $6.6x10^9$ gene number / g soil in the senescence stage.

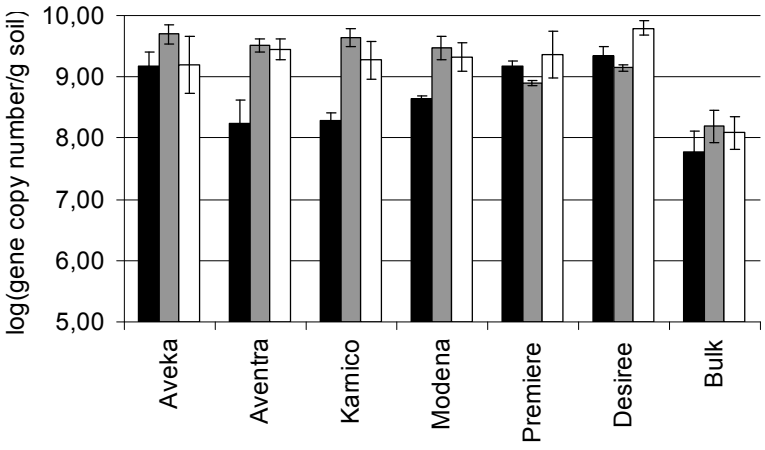


Figure 1 - Abundance of bacterial 16S rRNA genes in rhizosphere and bulk soil per growth stage in Buinen soil. Error bars indicate standard deviation. Black bars – young plant, grey bars – flowering, white bars – senescence.

Dynamics of bacterial diversity and community composition in bulk and rhizosphere soils as assessed by PCR-DGGE. For both bulk soil and rhizosphere samples, the bacterial PCR-DGGE patterns generated from all four replicate plots revealed high within-treatment similarities per cultivar and sampling moment (data not shown). This suggested generally low variabilities resulting from plot and sampling, DNA extraction, PCR amplification and DGGE.

The bacterial PCR-DGGE patterns generated from bulk soil revealed around 40% change over the season in both B and V field soils. In the B soil in June, the patterns were different from those before planting, as well as from those obtained in July and September (Fig 2A). Besides, the patterns generated from bulk soil collected one year later (unplanted soil) were again different from the foregoing (Fig. 2A). In the V soil, the bulk soil patterns showed only around 30% (gradual) change during the season, however around 80% change was observed when compared with the samples collected one year later (Fig 2B).

Rhizosphere effect - In all growth stages in the two soils, the rhizosphere bacterial PCR-DGGE patterns grouped apart from the corresponding bulk soil patterns (Fig. 3), thus indicating clear rhizosphere effects on bacterial community composition. This was confirmed by the fact that, in both soils, Monte Carlo permutations showed bulk soil

patterns to be significantly different from rhizosphere ones (P<0.05). Cultivar, growth stage and soil type also had an effect on the clustering of the patterns (Fig. 3A).

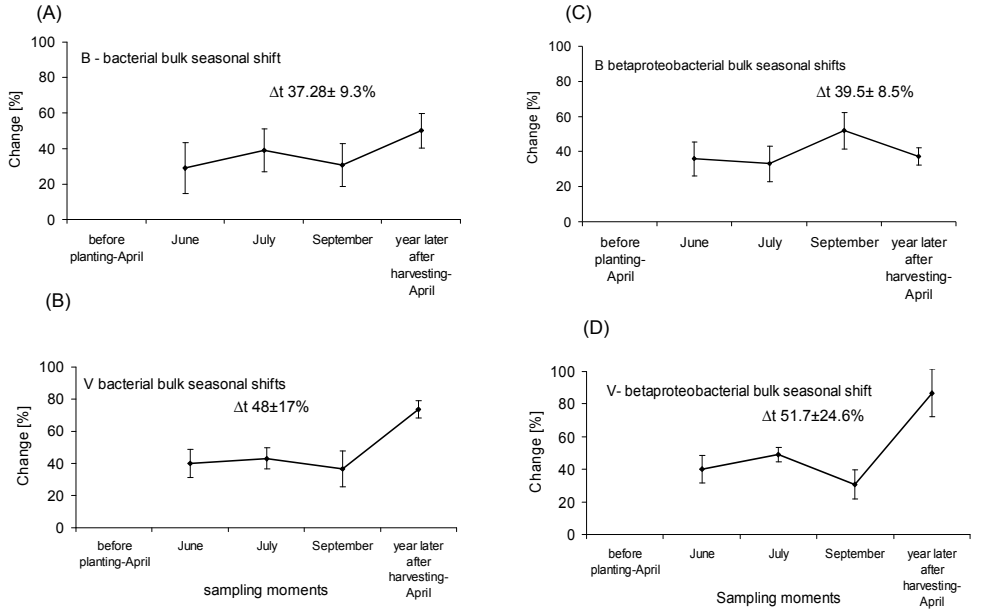


Figure 2 – Moving window analyses to evaluate the percentage of community change for bacterial communities in B soil (A) and V soil (B) and ß-proteobacterial communities in B soil (C) and V soil (D) through different sampling times. (Δt) the rate of change was calculated as the average of the respective moving window curve data points.

Cultivar effect – Five of the six bacterial PCR-DGGE patterns in the B soil potato cultivars (exception: cultivar A) grouped together in the young plant stage. However, in the flowering stage, cultivars K, M and P grouped together (P>0.05), while A, Av and D clustered apart, each as a separate unit (P<0.05). In the senescence stage, two main clusters were obtained (cultivars M, P and D in one hand, and cultivars A, Av and K on the other hand; Fig. 3B). In the V soil, the bacterial patterns of all six potato cultivars grouped closely together in the young plant stage and apart from the corresponding bulk soil patterns (data not shown). However, in the flowering stage, the patterns of cultivars A, Av and K grouped together, whereas those of cultivars M, P and D formed a separate cluster (Fig. 3C). In the senescence stage, the patterns of all cultivars again grouped together, except for cultivar A; all clustered away from the bulk soil patterns (data not shown).

Plant growth effect - In the B soil, plant growth effects were clearly observed for all cultivars, that is, the bacterial patterns in the rhizosphere shifted along time. For all cultivars, the patterns at the young and flowering stages clustered closer to each other than those at the senescence stage. In the V soil, plant growth stage also affected the

bacterial patterns. For all cultivars, the patterns in the senescence stage were significantly different from those at the young and flowering stages (P<0.05).

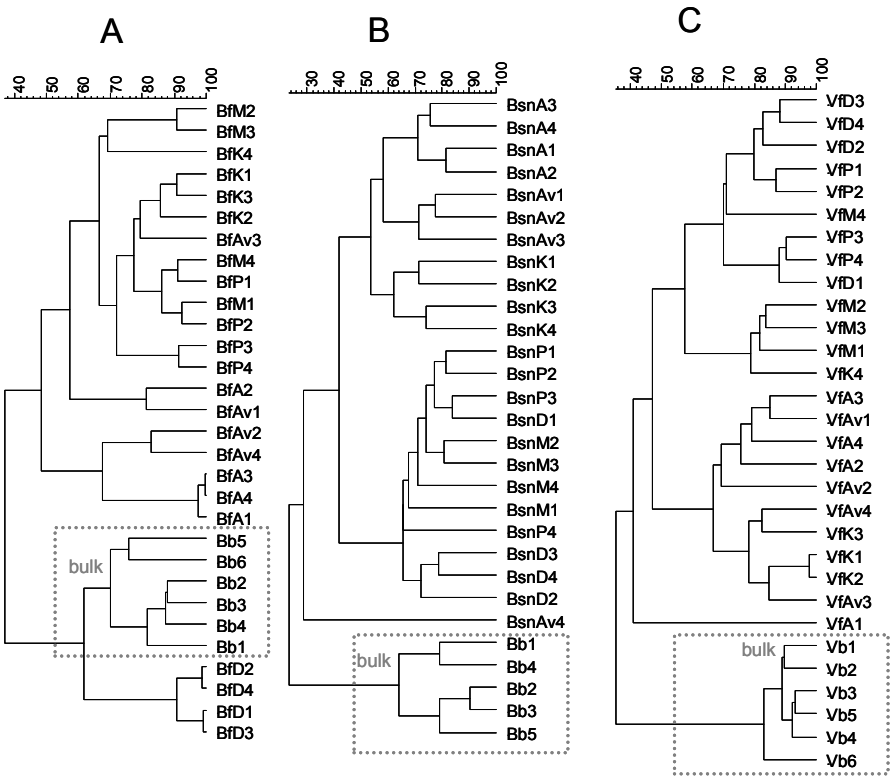


Figure 3 - Dendrograms representing the similarity of PCR–DGGE profiles gene rated with 16S bacterial DGGE system for B soil at flowering (A) and senescence stages (B) and for V soil, at flowering stage (C). [B] Buinen, [A] Aveka, [Av] Aventra, [K] Karnico, [GM] Modena, [P] Premiere, [D] Désirée, [V] Valthermond, [b] bulk soil, [f] flowering, [sn] senescence.

Dynamics of ß-proteobacterial communities in bulk and rhizosphere soils. The ß-proteobacterial communities in both bulk soils collected in June were different from those collected in July and September. For the B soil, these differences amounted to around 40% during the year. Although for the V soil a similar percentual difference occurred during the growth season, patterns of samples taken one year later in spring showed strong shifts in comparison to the samples taken before planting (Fig 2D).

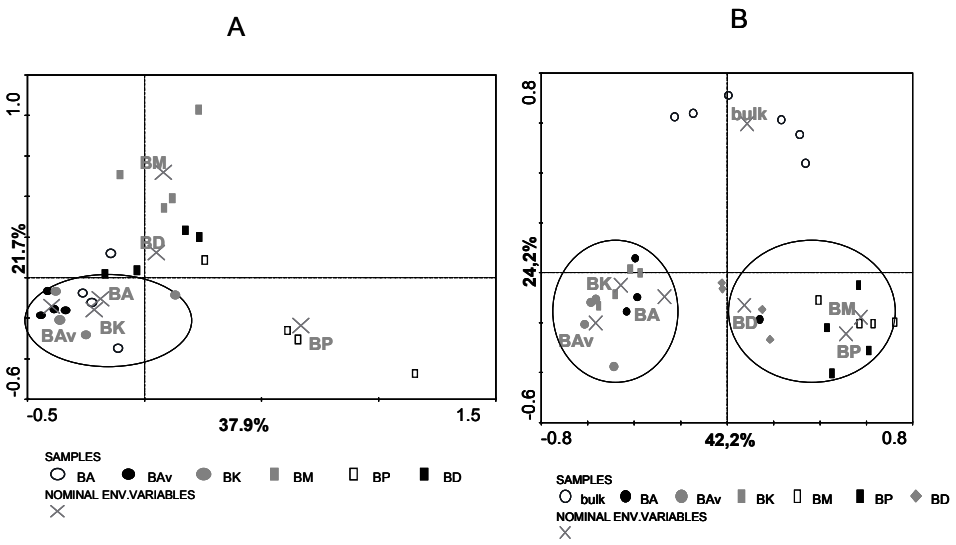
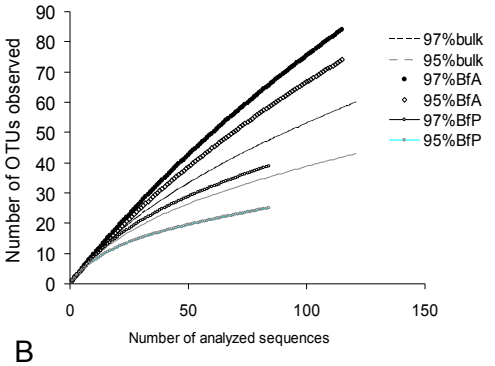


Figure 4 - Ordination biplot diagrams generated by redundancy analysis (RDA) of betaproteobacterial community in B rhizosphere and bulk soil, (A) flowering (B) senescence stage, ([B] Buinen, [A] Aveka, [Av] Aventra, [K] Karnico, [GM] Modena, [P] Premiere, [D] Désirée. The eigenvalues displayed on the diagram axes refer to the percentage variations of PCR-DGGE ribotypes; environment correlation accounted for the respective axis.

Rhizosphere and cultivar effects - In each plant growth stage in both soils, the rhizosphere-generated ß-proteobacterial PCR-DGGE patterns grouped apart from the corresponding bulk soil patterns (Fig. 4B). In the B soil, the patterns of all six cultivars tended to group together at the young stage, whereas effects of cultivar could be observed in the flowering and senescence stages. Specifically, in the flowering stage, cultivars A, Av and K grouped together; while M, P and D clustered apart (Fig. 4A). Monte Carlo permutation analysis showed that, at this growth stage, the two clusters were significantly different from each other ($P<0.05$). In the senescence stage, the clustering was comparable to that of flowering stage, with the patterns of cultivars P and M becoming more similar (Fig. 4B). In contrast, in the V soil the six cultivars showed clustering trends that were different form those in the B soil. In the young plant stage, the patterns of all cultivars except K and D grouped together. Monte Carlo analysis showed a significant difference of the K and D patterns from those of the other cultivars ($P<0.05$). In the flowering stage, the patterns of cultivars Av, M, P and D clustered together, whereas those of A and K clustered apart from this group. The patterns of the six cultivars grouped together in the senescence stage ($P>0.05$).

Finally, these analyses also showed a clear effect of soil type on the plant-associated betaproteobacterial communities, since the same cultivars showed community structures that were different between the two fields (data not shown).

A



B

	Sim.	Observed OTUs	Chao1 Avr.	Chao1 Low	Chao1 High
BfA	97%	84	218	151	353
	95%	74	198	132	336
BfP	97%	39	90	56	189
	95%	25	64	36	176
bulk	97%	60	124	87	209
	95%	43	79	56	146

Figure 5– Rarefaction curves of observed operational taxonomic units (OTU) at the "species" (97%) and "genus" (95%) levels for partial ß-proteobacterial 16S rRNA gene sequences (A) retrieved from B soil for cultivar P and D at flowering stage and bulk soil. The Chao1 richness estimations (B) with their respective low and high confidence limits (95%), as well as the number of OTUs determined with DOTUR for each case evaluated. [B] Buinen soil, [f] flowering, [A] Aveka, [P] Premiere.

Plant growth effect - A plant growth effect on the ß-proteobacterial communities was observed for all six cultivars in both soils. However, there were different trends over time in the two soils. In B soil at cultivars A and Av, the patterns in the senescence stage were significantly different from those in the young and flowering stages (which clustered together in RDA analysis). Monte Carlo permutation tests supported this contention, as the young stage patterns were statistically similar to those at the flowering stage ($P>0.05$). For cultivar K, the patterns of all three growth stages grouped together, whereas those of cultivars M, P and D showed, per cultivar, significant differences between the three growth stages ($P<0.05$).

In the V soil, the patterns of cultivars Av, K, and D clustered according to a similar pattern: the young and flowering stages clustered together, whereas the senescence stage clustered apart. Monte Carlo permutation tests showed these differences to be significant. While the patterns obtained at the flowering and senescence stages of cultivars A and M grouped together, the young stage grouped apart from this combined cluster. Contrary to what was seen in the B soil, the patterns obtained during growth of cultivar P grouped closely together, which was supported by Monte Carlo analysis ($P>0.05$).

Analysis of ß-proteobacterial clone libraries. Since the ß-proteobacterial PCR-DGGE analyses had revealed clear differences between the cultivars with high and low starch content tubers at flowering stage, one high-starch and one low-starch cultivar (respectively A and P), in addition to corresponding bulk soil, were selected for the

construction of three ß-proteobacterial 16S rRNA gene clone libraries. After quality and chimera checks, totals of 121, 115 and 84 sequences were obtained from bulk soil and cultivar A and P rhizospheres, respectively. Shuffling analysis [49] showed that each library was significantly different from any of the other ones. Rarefaction curves were then generated to assess the depth of sampling and the richness of the libraries, using 97 and 95% cut-off criteria for the grouping of OTUs at "species" and "genus" levels, respectively. None of these curves reached the plateau level. Although we did not sample to saturation, both rarefaction and CHAO1 analysis showed significant cultivar effects on the ß-proteobacterial community, with the library obtained from cultivar A being the most diverse, based on rarefaction curves and non-parametric CHAO1 richness estimates (Fig. 5b). Besides, these analyses showed the cultivar P library to have the lowest richness, and the bulk soil library to be intermediate.

Table 1 Comparison of clone libraries (RDP analysis)

		%BfP	%BfA	%Bfbulk
family	***Comamonadaceae***	20.2	25.2	0.8
genus	*Variovorax*	10.7	7	
	unclassified Comamod.	7.1	13.9	0.8
family	***Oxalobacteraceae***	14.3	11.3	0.3
genus	*Massilia*	6	1.7	0.8
genus	*unclassified Oxalobact*	7.1	7	
family	***Nitrosomonodales***	4.8	2.6	16.5
genus	*Nitrosospira*	4.8	2.6	16.5
family	***Alcaligenaceae***	4.8	14.8	0.8
genus	*Achromobacter*	1.2	11.3	0.8
genus	*Derxia*	2.4		
family	***Incertae sedis 5***	8.3	7	7.4
family	***Burkholderiaceae***	28.6	15.7	28.1
genus	*Burkholderia*	28.6	14.8	27.2
	unclassified Betaproteo	15.5	3.5	32.2

All sequences, even those that were similar to database entries at low levels of similarity (i.e. <97%), were affiliated with ß-proteobacterial 16S rRNA gene sequences (Fig. A1). Using the RDP library comparison at a confidence threshold of 80%, the majority of the sequences was affiliated to recognized classes of the *ß-proteobacteria* [4], whereas the remainder was affiliated with unclassified *ß-proteobacteria* ([15-30%], Table 1). In addition

to differences in richness, there were significant differences in community make-up, with respect to the prevalence of particular groups, between the two rhizospheres and the bulk soil. For instance, sequences assigned to the family *Comamonadacea*e accounted for 25.2 and 20.2% of the clones from the rhizosphere of cultivars A and P, respectively, whereas this group made up only 0.8% of the amplicons generated from the bulk soil. Within the *Comamonadacea*e, the genus *Variovorax* was found to be abundant in both rhizospheres, at respectively 50 and 28 % of the *Comamonadacea*e for cultivars P and A, respectively. In contrast, this genus was completely absent from the bulk soil library. The second highly abundant family in the rhizosphere was the *Oxalobacteriaceae*. The family *Alcaligenaceae* was also selected by both rhizospheres, but mostly so by cultivar A. In particular, the genus *Achromobacter* ($P < 0.0003$) was significantly dominant in cultivar A. On the other hand, the genus *Nitrospira* was more abundant in the bulk soil than in the rhizosphere. Finally, the *Burkholderiaceae* were found to be equally (28%) abundant in the cultivar P and bulk soil libraries, whereas it amounted to 14% of the cultivar A library (Table 1).

Quantification of *Variovorax spp.* using *asfA*-based qPCR. Given the abundance of *Variovorax* spp. in the rhizosphere libraries and to the importance of *Variovorax paradoxus* in the desulfonation process [45], the abundance of *asfA* genes was measured by qPCR to assess the putative effects of rhizosphere and cultivar on the desulfonation process. The *asfA* gene copy numbers did not change in the B bulk soil over the growth season. In contrast, in the V bulk soil a rapid increase in *asfA* gene numbers was observed from June to July (Fig. A2-B), while these numbers did not show a change between July and September.

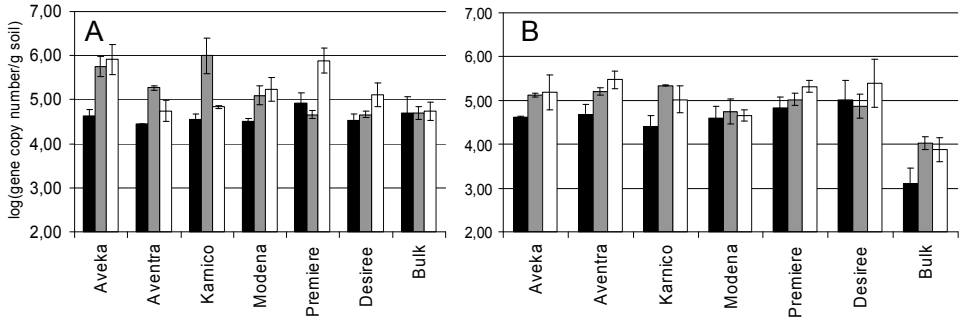


Figure 6 Abundance of *Variovorax asfA* genes in rhizosphere of different potato cultivars and corresponding bulk soil, at different growth stages in B soil (A) and V soil (B). Error bars indicate standard deviation. Black bars – young plant, grey bars – flowering, white bars – senescence.

In both soils, a rhizosphere effect on the *Variovorax asfA* gene abundance was clearly observed, since this abundance was much higher in the rhizospheres than in corresponding bulk soils. This rhizosphere effect was significant in all growth stages of all cultivars, except for cultivar A at the young stage in the B soil (Fig 6A). Moreover, an effect of cultivar type on the dynamics of the *asfA* gene abundances was also observed. In B soil, this abundance increased significantly for cultivars A, Av, K and M from the young to flowering stages, and for cultivars P and D from the flowering to senescence stages. In the

V soil, the *asfA* gene abundances with cultivars A, Av and K showed similar trends, i.e. increases from the seedling to flowering stages (P>0.05) and no significant differences for cultivars M, P and D.

In the B soil, the *asfA* gene abundances with the cultivars producing high-starch-content tubers (A, Av, K) showed trends that were different from those with low-starch-content tubers (P and D). Besides, the *asfA* gene abundances with cultivar M showed different trends depending on the soil: in B soil, these were between those of parent cultivar K and low-starch-tuber cultivar P (Fig 6B).

Discussion

In this study, we assessed the dynamics in the abundance and community structure of selected soil bacterial communities as a function of plant cultivar, growth stage and soil type, using six potato cultivars in two soils of different texture. Clearly, effects of soil type, cultivar and growth stage on plant-associated communities were measurable by our techniques. Soil microbial communities can be affected by many factors, such as soil characteristics, environmental conditions and crop management strategies like rotation and crop residue removal [15, 33]. Accordingly, community changes were observed over time in the bulk soil of both fields. Microbial community changes in bulk soil between the pre-planting and young plant stages have previously been shown [26]. Interestingly, in our study drastic changes were observed in the V soil after one year, which could have been caused by the heavy water accumulation in this field the year after harvesting, due to its soil type. Thus, the changes in bacterial community structures might be explained by differences in water contents and oxygen limitations in case of water logging.

The changes in community structure were not associated with changes in bacterial abundances, except for the beginning of the growing season (before planting). This indicates that agricultural practices used when preparing the soil for planting (ploughing, fertilization) might reduce bacterial abundance, regardless of soil type.

When analyzing the effect of plants on bacterial abundance and community structure, significant differences were observed in both fields at all growth stages. The increase in abundance in the rhizosphere in comparison to bulk soil was expected, as the presence of substrates released by plant roots through exudation would have a direct effect on bacterial abundance. In the V soil however, the bacterial abundance remained roughly constant over the growth season. It is possible that, due to its higher nutritional status (organic matter content and nutrients), the bacterial abundance in V soil was affected to a much lower extent by root exudates.

Moreover, the rhizosphere bacterial and ß-proteobacterial communities were also significantly different from their respective bulk soil. Rhizospheric microbial communities are known to be affected by complex interactions among soil types, plant species (genotypes), and growth [29, 43]. Previous studies indicated that rhizosphere populations decline as the plant matures [2, 43], whereas other studies showed that microbial diversity increases with plant age [22, 38]. Even though, in our study, the rhizosphere did not always harbor higher bacterial abundances than corresponding bulk soils, this does

not imply that a rhizosphere effect was absent. In fact, the influence of roots on bacterial populations in the rhizosphere may be small or ephemeral, but still present.

We obtained evidence for a particular cultivar effect, which was especially evident for the ß-proteobacterial community structure. The usual PCR biases [1] may affect the frequency and/or presence of sequences in DGGE or clone library analyses. Preferential annealing is one such bias, and selection of the most abundant sequences at the expense of low-abundance ones is another one. These less-dominant groups might become apparent in PCR-DGGE only if specific primers are used to reduce the complexity, which improves resolution of the rarer types. The analysis of the *ß-proteobacteria* allowed us to look into such a less dominant group, which has previously been shown to be around 4-16% in a soil community [50]. Interestingly, the observed cultivar effect, which correlated with the tuber starch content and root development, also correlated with growth stage, being absent in the young plant stage and becoming more evident in the flowering stage. In the case of B soil, the cultivar effect became even stronger at senescence stage, for both bacterial and ß-proteobacterial community structures. Regarding the V soil, a clear separation between cultivars was observed only at the flowering stage, for total and ß-proteobacterial communities, but the correlation between tuber starch content and community structure was observed mainly for bacterial communities. As mentioned above, the difference per soil can be a result of soil characteristics. Different cultivars with different growth rates and root development are likely to release organic compounds to different extents, and the bacterial populations in the rhizospheres of the two cultivar groups with different tuber starch contents possibly consisted of species that utilize different carbon sources. Thus, in response to changing root exudation patterns, the microbial community composition in the rhizosphere will also change with time, thus varying during the life cycle and seasonal response of plants [10]. Also, the amount and type of compounds in root exudation patterns might show genotype-specific variations. In some cases, plant species type may have a greater influence on microbial community composition than soil type [16, 55], whereas the effect of soil type on the community may be superior to the effect from plant species type in others [6, 11, 48]. A plant genotype-specific selectivity of plant roots on rhizobacterial community structures was also observed in previous studies [11, 12, 35, 41, 53].

In order to better understand the effect of the differences in plant physiology (related to tuber starch content and root development) on rhizospheric bacterial diversity, clone libraries from two cultivars growing in B soil during flowering stage were compared to the respective bulk soil. Surprisingly, the three libraries varied remarkably in diversity estimates of the *ß-proteobacteria*. Specifically, the rhizosphere of the cultivar with high-starch-content tubers showed the highest ß-proteobacterial diversity, whereas the communities associated with the low-starch-tuber cultivar was the least diverse. It is reasonable to expect that the physiological changes that lead to plants with different tuber starch contents and growth rate lead to changes in the quality and/or quantity of the exudates released by the roots. In this context, one could speculate that high-starch-content-tuber/ slow-growing plants would show a more diverse pattern of organic compounds being released by the roots, which would sustain higher ß-proteobacterial diversities. On the other hand, the low diversity observed in the rhizosphere of the low-starch-tuber cultivar with fast growth rate could be due to simpler exudation patterns. For

instance, differences in the abundances of *Achromobacter* were observed. *Achromobacter piechaudii* has been shown to contain ACC deaminase activity, reduce the level of ethylene [32] and increase resistance to salt [31], flooding [17] and pathogen stress [54]. Despite the differences in diversity estimates, the rhizosphere communities also showed some general trends, as several genera found in the clone libraries are known as plant-growth promoting bacteria. *Burkholderia phytofirmans,* which was found in both rhizosphere samples, can also reduce the level of ethylene [47]. *Comamonadaceae* and *Oxalobacteriaceae,* which were highly dominant in both rhizospheres, were found to be preferentially associated with mycorrhizal roots in *Medicago truncatula* [39, 40]. The presence of arbuscular mycorrhizal fungi was also shown in most of the rhizospheres independent of cultivar type (E. Hannula, pers. comm.). Within the family *Comamonadaceae*, the genus *Variovorax* was dominant, accounting for up to 50% of the *Comamonadaceae* clones obtained from the cultivar with high starch content, whereas it was completely absent from the bulk soil. Moreover, according to our results, the species *Variovorax paradoxus,* which has been found to be the key desulfonating species in the wheat rhizosphere [45] seems to be an important rhizobacterium under potato as well, indicating the relevance of this group for potato plants. It will be interesting to determine whether these results apply for other crops as well. Additionally, the role of the 7-14% unclassified *Comamonadaceae* in the rhizosphere is unclear, but they might be involved in desulfonation. Schmalenberger et al. [46] showed that related species such as *Polaromonas* and *Acidovorax* are involved in desulfonation in the wheat rhizosphere.

A remarkable observation was the relative abundance of the *asfA* gene in the rhizospheres of V soils versus that in bulk soil, whereas this difference was clear after young plant stage between B rhizospheres and bulk soils. Regardless of soil type, the abundance of *asfA* among the cultivars varied during growth, with high-starch-tuber cultivars increasing the number of *asfA* gene copies as they matured from young plant to flowering, whereas for the low-starch-tuber cultivars, the *asfA* gene copy numbers remained rather stable throughout the season. The effect of the physiology of potato plants that produce tubers with different starch content on the rhizospheric *V. paradoxus asfA* gene numbers possibly overcame the soil type effect, even though the community structure of the *ß-proteobacteria* associated with these soils were different. More studies are needed to confirm the role of *V. paradoxus* in desulfonation in the potato rhizosphere, as well as the role of other potential desulfonating bacteria.

In our study, the genetically modified cultivar M fell in the value range of the other cultivars, for all measured variables. Thus, cultivar M did not have any outstanding effect on bacterial community structure and abundance. Previously, Milling et al. investigated the effects of transgenic potato (producing tubers with altered starch composition) on the composition of bacterial and fungal communities in the rhizosphere [36]. They compared a parent, transgenic line and another non-transgenic cultivar over three seasons. They also did not observe any significant influence of the modification on dominant members of the rhizosphere bacterial communities. Based on our results, we conclude that, although the cultivar M patterns incidentally showed differences from those of cultivar K, these were still similar to those of the other cultivars (the baseline), in particular P and D. Interestingly, all three cultivars produced high-starch-content tubers, much like cultivar M.

Our study showed a strong effect of plant growth stage as well as soil type on the bacterial communities that associated with potato. The potato cultivars grouped in accordance with the starch content of the tubers and hence the plant-specific variations in the rhizosphere bacterial communities correlated with effects on, e.g., the rhizosphere or root architecture, exerted by tuber starch content. The genus *Variovorax paradoxus* was found to be abundant in the potato-associated bacterial communities and asfA genes, involved in desulfonation, were abundant in several cases. Moreover, the genetically modified cultivar fitted the baseline, which fluctuated between low and high starch content potato cultivars.

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